DEVELOPMENT OF AN ASSAY FOR SPHINGOMYELINASE D PRODUCTS IN VENOM FROM *LOXOSCELES RECLUSA* AND OTHER SPIDERS

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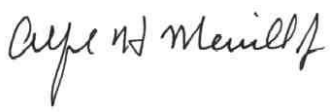
Approved by



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ABSTRACT

The venoms of *Loxosceles reclusa*, the brown recluse spider, and some other *Loxosceles* species cause dermonecrotic lesions—a dying of the flesh—and in severe cases, systemic injury and death. Since this type of injury results from a bite, diagnosis can be cryptic unless the culprit spider is available to be identified by an expert. The distribution of *Loxosceles reclusa* is also not well known, which further complicates diagnosis. These spiders are of additional interest because one of the agents in the venom is a sphingomyelinase D (SMase D) that cleaves the head group of sphingomyelin (SM) and causes intramolecular transphosphatidylation to produce ceramide 1,3-cyclic phosphate (Cer(1,3)P), a lipid that is not known to be present in humans otherwise. Therefore, a simple assay to analyze the presence of SMase D, by either activity assays or detection of its product Cer(1,3)P, could aid in identification of spider bites and possibly facilitate development of a treatment. A key reagent for such studies is Cer(1,3)P which is currently not commercially available, so we have developed a method for its synthesis and identified conditions for distinguishing Cer(1,3)P from the substrate SM and alternative hydrolysis products (ceramide-1-phosphate and ceramide) by thin-layer chromatography. The availability of this chemical and its synthesis procedure enables an assay of SMase D for better characterization of this important component of brown recluse spider venom (and possibly other organisms). Moreover, the assay could be used to search for enzymes that hydrolyze Cer(1,3)P, and such enzymes might prove useful in the development of a treatment for these wounds.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

C12	Lauroyl
C24	Lignoceroyl
Cer	Ceramide
Cer(1,3)P	Ceramide 1,3-cyclic phosphate
Cer1P	Ceramide-1-phosphate
CerK	Ceramide kinase
DCCD	N,N'-dicyclohexylcarbodiimide
DMF	N,N-dimethylformamide
ELISA	Enzyme-linked immunosorbent assay
LPA	Lysophosphatidic acid
LPC	Lysophosphatidylcholine
MeOH	Methanol
MS	Mass spectrometry
NBD	Nitrobenzoxadiazole
PLD	Phospholipase D
PMN	Polymorphonuclear
SM	Sphingomyelin
SMase C	C-type sphingomyelinase
SMase D	Sphingomyelinase D
SMS	Sphingomyelin synthase
TLC	Thin-layer chromatography

INTRODUCTION

Brown recluse spiders, namely *Loxosceles reclusa,* are often said to be responsible for the majority, if not all, of the necrotic wounds caused by spiders in North America.[1] Brown recluse spiders are most highly concentrated in tropical areas of South America, and they or related spiders have been found worldwide, particularly in the Western Hemisphere.[2] Nonetheless, there is an ongoing discussion[2] of whether all necrotic wounds that appear in medical clinics are due to loxoscleism (a bite by a spider from the genus *Loxosceles*) because there are many cases in which this spider's presence has not been documented.

Distribution of the *Loxosceles* spiders is widespread, yet the exact distribution is not well known. Across South America, most species that are known to cause necrosis are *Loxosceles laeta*, *L. intermedia*, and *L. gaucho*, with *L. laeta* being the most toxic.[3] Several of these species endemic to South America, particularly *L. laeta,* are shown to be larger and are thus supposed to have more deleterious venom than *Loxosceles* spiders endemic to North America.[4] *Loxosceles* is not well documented across the rest of the world.

Loxosceles spiders are nocturnal and prefer to hide in small nooks. In unnatural environments, *Loxosceles* spiders prefer mediums of construction materials, wood, and paper.[5] These spiders are characterized by their reclusive tendencies of sheltering themselves from humans and are known as "araña de detrás de los cuadros" (the spider behind the picture) or "araña de los rincones" (spider of the corners) in South America.[6]

A violin pattern on the dorsal surface of the cephalothorax of *Loxosceles* spiders is a definitive method of their identification (Fig. 1).[2] Problems of misidentification typically arise in lack of expertise. The violin pattern may perhaps be an overly simplified identifier and holds as an accurate identifier for most, but not all, *Loxosceles* spiders. A diagnostic feature that allows us

to better corroborate a spider's characterization as *Loxosceles* is that *Loxosceles* spiders have six eyes arranged in non-touching pairs in a U-shaped pattern (Fig. 2).[2]

Figure 1. *Loxosceles reclusa* spider. Also known as the brown recluse or "violin spider," *Loxosceles reclusa* has a characteristic violin-shaped patch on its cephalothorax. The spider has long, slender legs and is light brown in color. This image is from the Missouri Department of Conservation (https://nature.mdc.mo.gov/discover-nature/field-guide/brown-recluse-violin-spider).

Figure 2. Anterior view of *Loxosceles reclusa*. This spider has a dorsal brown violin pattern with six eyes situated in non-touching pairs in a U-shaped pattern. The violin pattern can show variance among species. The eye pattern can be used as a more confident measure of identification since it is non-varying among *Loxosceles* species. This image is from Swanson & Vetter (2006).[2]

Loxosceles[2] and *Sicarius*[7] spiders, both of the family Sicariidae, are the only spiders known to result in skin necrosis from spider bites. Bites commonly happen in instances in which the spider is pressed again the skin. Bites by *Loxosceles* spiders have a relatively low risk, as there can be as many as thousands of *Loxosceles* spiders in an area with no medical incidents reported.[8]

Though toxins are of the same constituency in males and females, females carry a more concentrated venom.[9] Spiders quite likely deliver varied amounts of venom in defensive biting, altering the severity of the injury from the bite.[10] Amount of venom delivered also likely varies with prey type.

Misidentification and Misdiagnosis

Over the past few decades, bites from spiders across various genera, such as wolf spiders, funnel-weaving spiders, and orbweavers, have been placed under the term "necrotic arachnidism."[2] The majority of these identifications have been false due to lack of information and evidence of the culprit spider, making accurate diagnosis difficult.

Since common diseases can mimic spider bites, *Loxosceles* bites can only be identified with true confidence if the spider that produced the bite can be identified; otherwise the diagnosis may not be accurate.[2]

The most common and accurate diagnosis of spider bites is done by correctly identifying the spider responsible for the bite. Diseases commonly misdiagnosed as loxoscleism include diabetic ulcer, chemical burn, squamous cell carcinoma, syphilis, toxic epidermal necrolysis, Lyme disease, and anthrax.[11–13] Since myriad diseases mimic loxoscleism and distribution of

Loxosceles spiders is not well documented, diagnosis cannot be confident if the culprit spider is not identified by an expert.

Diagnosis is hugely dependent upon proper characterization of the responsible spider; however, a venom test has been developed. This test uses an enzyme-linked immunosorbent assay (ELISA) which is an assay technique to detect and quantify peptides, proteins, antibodies, and hormones.[14] There is no assay commercially available to humans, but in rabbits, venom was recoverable both form hair/skin and biopsy specimens with this assay.[14]

In less serious cases, a bite by a *Loxosceles* spider can result in a mild urticarial reaction, producing swollen, pale red bumps on the skin.[15] In more severe cases, the bite area turns a pale color, and the area adjacent to the bite turns red and edematous, with mild to severe pain (Fig. 3).[3] While the initial bite can be painless at first,[3] sharp pain follows than can evolve into a burning sensation.[16] In the days following the initial bite, the wound typically turns a blue-purple color and has a hard, sunken center, with sloughing occurring.[3] In the most severe of cases, ulcers up to 40 centimeters in diameter can form and extend into deep muscles.[17]

Figure 3. *Loxosceles reclusa* bite on a human. This is an example of a serious, advanced wound. The ischemia causes a literal dying of tissue, or necrosis, around the bite. *Loxosceles* bites are further characterized by dermal edema, vasodilation, hemorrhage, and intravascular coagulation. This image is from Malaque et al. (2015).[18]

Other common side effects of loxoscleism include weakness, fever, malaise, nausea, vomiting, thrombocytopenia, disseminated intravascular coagulation, and hemolytic anemia.[3] Renal failure can be induced by myonecrosis and rhabdomyolysis resulting from a *Loxosceles* bite.[19] *Loxosceles* bites have been shown to result in death in South America,[3] but in North America, there are no definitively proven deaths.[20]

Treatment

Currently, no effective evidence-based therapy for loxoscleism exists.[2] Minimal treatment includes elevation, immobilization, application of ice, and local wound care. Additional therapies that have been used include antihistamines,[14] antibiotics, dextran, glucocorticosteroids,[21] vasodilators,[22] dapsone, surgical excision, and antivenin.[14]

Dapsone comes highly recommended because of the prominent role of polymorphonuclear (PMN) leukocytes in the pathophysiology of the injury. Dapsone has been shown to reduce skin lesion size in guinea pigs injected with brown recluse spider venom.[23] However, no human study shows that dapsone is an effective treatment for *Loxosceles* bites in humans.[24]

Loxosceles laeta rabbit antivenom is used in South America.[3,25] Antivenoms against *Loxosceles* venoms prevalent in the Americas were able to fully neutralize their respective venoms upon a one-hour incubation.[26] Current therapies are minimally effective in treatment of loxoscleism, as there is a deficiency in the understanding of the mechanism(s) of *Loxosceles* spiders' venom.[27]

Mechanism

The main components of the venom that are thought to be responsible for loxoscleism are phospholipase D (PLD) toxins which function by attacking the distal phosphodiester bond in phospholipids and subsequently freeing the polar head group.[28,29] The prototype activity ascribed to the venom is a sphingomyelinase D (SMase D) that, by analogy to most PLD, would be expected to cleave sphingomyelin (SM) into headgroup choline and ceramide-1-phosphate (Cer1P) (Figs. 4 & 5). An additional activity that has been found is the release of choline from lysophosphatidylcholine (LPC) resulting in lysophosphatidic acid (LPA).[30,31,32]

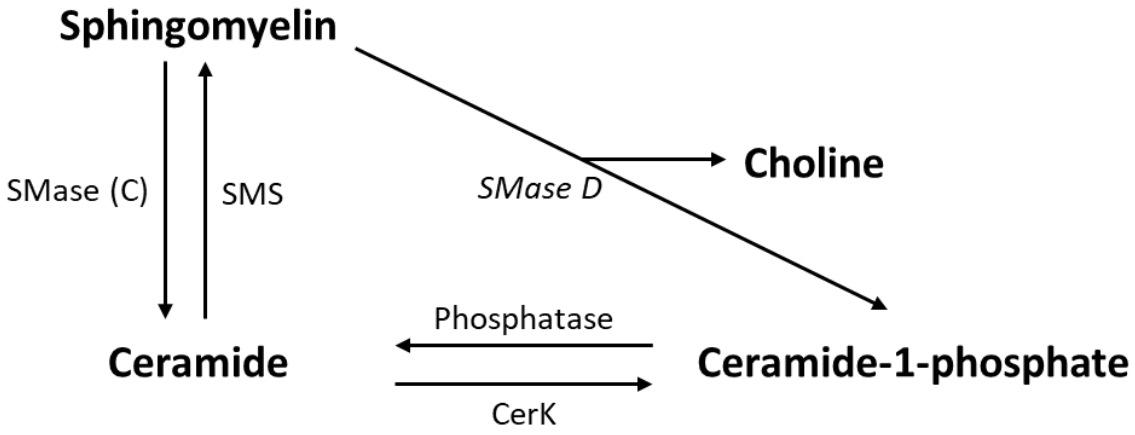

Figure 4. Cer1P synthesis pathways. Direct synthesis of Cer1P can occur via SMase D acting on SM, with choline as a byproduct. Indirect synthesis of Cer1P can occur through the activities of both a C-type sphingomyelinase (SMase (C)) and ceramide kinase (CerK), with a ceramide intermediate. SMS, sphingomyelin synthase. This figure is adapted from Rivera et al. (2015).[33]

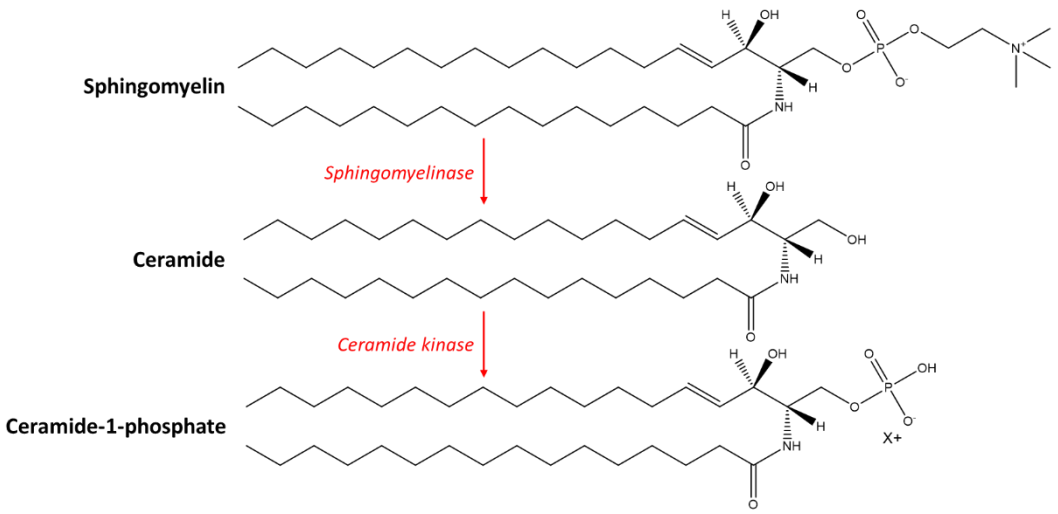

Figure 5. Biosynthesis of Cer1P. Starting with SM, Cer1P can be synthesized with an intermediate of ceramide.

The enzymatic activities of *Loxosceles* venom include esterase,[34] ATPase,[35] sphingomyelinase D,[36] hyaluronidase,[37] alkaline phosphatase,[37] but not phospholipase A/C,[35] phosphodiesterase,[38] or collagenase.[35] *Loxosceles* venom may or may not have protease activity. Proteases are present in *Loxosceles* gastric contents and are able to cleave native collagen, fibrinogen, fibrin, elastin, gelatin, and fibronectin. In a rabbit model, gastric contents cannot cause necrosis, but it is possible that gastric contents could act with sphingomyelinase activity from the venom.[39]

Until recently, formation of Cer1P, and possibly LPA, were rationalized to account for the disruption of cell behavior because these are highly bioactive (signaling) lipids; however, studies of the cloned and expressed enzyme have reported that instead of the production of Cer1P as presumed, SMase D actually causes intramolecular transphosphatidylation to produce ceramide 1,3-cyclic phosphate, Cer(1,3)P (Fig. 6).[40] Studies of crude *Loxosceles* venom have also determined that the major product of SM cleavage is Cer(1,3)P.[40]

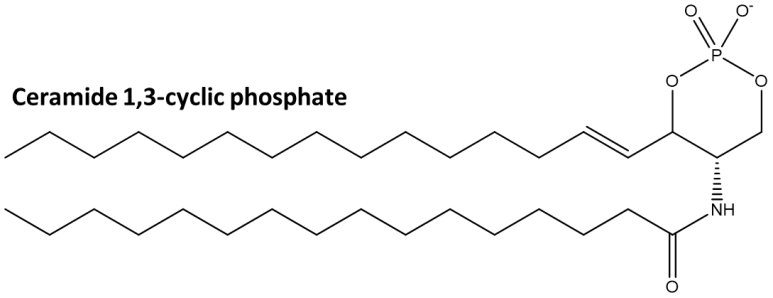

Figure 6. Ceramide 1,3-cyclic phosphate. Cyclic form of Cer1P, produced by the enzyme sphingomyelinase D.

Therefore, production of Cer(1,3)P is highly likely to be a marker, and possibly even the cause, of the necrotic lesions associated with brown recluse spider venom. Thus, if it were possible to routinely assess this activity in clinical cases of necrotic wounding, it might be clearer if all (or most) necrotic wounding is caused by this spider. However, additional spiders could be involved too because comparisons of the genomes of other spiders have suggested that other spiders might also produce a SMase D.[41] *Sicarius* spiders, which are in the Sicariidae family with *Loxosceles* spiders, contain PLD enzyme toxins.[7]

Phylogeny

Spider bites from orb-spinning spiders do not have sphingomyelinase D, and no longer are they deemed to cause necrosis.[2] *Sicarius* remains the only other spider shown to contain significant levels of sphingomyelinase D in its venom. This spider, however, has less significance medically since it primarily resides in South Africa and limits human contact. The instance of dermonecrosis in bites has become increasingly unlikely, though, as *Sicarius* spiders have lost or significantly reduced their ability to produce sphingomyelinase D.[2] Over the decades, the realm of necrotic bites has boiled down to loxoscleism.

Research in the Merrill laboratory has provided reason to believe that the *Kukulcania hibernalis* spider very likely possess sphingomyelinase D activity, evidenced by disappearance of SM and production of a product with a higher mobility on TLC (unpublished findings). However, the lack of a standard for Cer(1,3)P precluded identification of the product as Cer1P or the cyclic metabolite now known for the brown recluse spider venom.

The pathogenic bacterium *Clostridium perfringens* has been shown to worsen the effects of lesions,[42] and mammalian assays of SMase D active cDNA expression products demonstrate that SMase D is the culprit of lesion formation.[43,44] There is speculation of an evolutionary relationship between spider and bacterial SMase Ds.[45] SMase D activity is also known in the pathogenic bacteria *Corynebacterium pseudotuberculosis*,[45] *C. ulcerans, Archanobacterium haemolyticum,* and *Vibrio damsela*.[46,47] *Loxosceles, C. pseudotuberculosis,* and *A. haemolyticum* SMase Ds are similar in molecular weight (30-35 kDa), isoelectric point and charge, and share conserved amino acid residues in the N-terminus.[31,47–49]

BLAST searches have shown the presence of homologous enzymes to SMase D in the fungi *Aspergillus* and *Coccidioides*,[50] and this has been confirmed experimentally in *A. flavus*.[51] A common motif at the C-terminal end of SMase D was identified, supporting the inference about the origins of these enzymes stemming from the broadly conserved glycerophosphoryl diester phosphodiesterase family.[52] By inferring that the function of the C-terminal conserved motif is to stabilize the internal structure of the SMase D TIM barrel, it appears that SMases D are widely represented in several genera and can act as a pathogenic effector.[51]

Research Thrusts

The goal of this study is to develop a relatively easy method to analyze Cer1P, Cer(1,3)P, and ceramide (Cer) to determine which products are made by different organisms, beginning with the brown recluse spider SMase D and eventually extending to other organisms. There are several reasons why this assay could be useful. A simple but analytically rigorous assay for the SMase D activity found in brown recluse spider venom might enable assessment of whether wounds that are ascribed to a bite from this spider are, indeed, associated with the presence of this activity. The assay could also be used to determine if other spiders have similar activity (i.e., the capacity to hydrolyze SM to Cer(1,3)P), and if so, this might explain why there is controversy over whether or not the brown recluse spider is responsible for all of the necrotic lesions that are clinically observed. Furthermore, if there is an association between Cer(1,3)P and necrotic lesions, an assay for Cer(1,3)P might facilitate development of a treatment to remove this product (such as an enzyme that would cleave one or both ester bonds) to decrease the extent of necrosis and allow for better and faster recovery of the affected individuals.

METHODS AND MATERIALS

Sources of Reagents

All of the sphingolipids (C12- and C24- Cer and Cer1P) and fluorescent analogs (NBD-Cer, NBD-Cer1P, and NBD-SM) were obtained from Avanti Polar Lipids (Alabaster, Alabama). The C24-Cer(1,3)P was synthesized from C24-Cer1P by modification of a published method[53] (see below).

The HPLC-grade organic solvents (chloroform, # EM-CX1050 and methanol, # EM-MX0475) were obtained from VWR (West Chester, PA); ACS grade acetic acid (# A38C-212) was obtained from Fischer (Pittsburg, PA); and all other solvents were analytical grade. N,N'-dicyclohexylcarbodiimide and iodine were from Sigma-Aldrich (St. Louis, MO). The thin-layer chromatography (TLC) plastic sheets (Silica gel 60) were from EM Science (Darmstadt, Germany).

The SMase D from recluse spider venom was obtained via Chuck Kristensen at Spider Pharm (Yarnell, Arizona).

Pilot Synthesis of Cer(1,3)P

A pilot synthesis of Cer(1,3)P was conducted following the reaction conditions described by Boudker and Futerman[53] for the synthesis of C6-NBD-Cer(l,3)P. It was conducted using C12- (lauroyl-) and C24- (lignoceroyl-) CerlP (Avanti) (~1 μmol) incubated with a 1.5-fold molar excess of N,N'-dicyclohexylcarbodiimide (DCCD) in 2 mL of N,N-dimethylformamide in 13 x 100 mm glass screw-capped test tubes with a saturated atmosphere of N_2. The mixture was turbid, so the reaction time was increased to 4 days (instead of 1-2 days) at room temperature, with sonication, as well as heating the tube under warm water, to attempt to bring all of the

reactants into solution (this appeared to help, but the mixture was always somewhat cloudy). Afterward, the N,N-dimethylformamide was dried under a stream of N_2, and the products were dissolved in chloroform and methanol (2:1, *v*/*v*) and extracted with essentially as described by Bligh and Dyer.[54] The solvents from the product extraction were removed under a stream of N_2, redissolved in 0.5 mL of chloroform and methanol (2:1, *v*/*v*), and aliquots were examined by TLC on silica gel plates using chloroform:methanol:acetic acid:15 mM aqueous $CaCl_2$ (60:35:2:4, *v/v/v/v*) as the developing solvent. After the solvents had dried, the plate was placed in a tank with saturating I_2 vapor for several hours to visualize the hydrophobic compounds, and then the plate was examined. Based on Boudker and Futerman,[53] the R_f for Cer(1,3)P should be higher than starting Cer1P and would be predicted to be below the unreacted DCCD and byproduct N,N'-dicyclohexylurea.

Optimized Synthesis of Cer(1,3)P

After testing several solvents as alternatives for DMF, pyridine was selected as the best because it has been used in other DCCD coupling papers, and all of the reagents for this synthesis were readily soluble in it. In addition, since C24-Cer1P was readily soluble in pyridine and was available in larger quantity, the subsequent synthesis was conducted with it alone. Approximately 1 μmol of C24-CerlP (Avanti) in pyridine was mixed with another 0.5 mL of pyridine containing ~2 μmol of DCCD in a screw-capped test tube, then the air was replaced by N_2, and the reaction mixture was left for 2 days at room temperature with gentle rocking. Afterwards, the pyridine was dried under a stream of N_2 and the products were dissolved in chloroform and methanol (2:1, *v*/*v*) and extracted with essentially as described by Bligh and Dyer.[54] The solvents from the product extraction were removed under a stream of N_2, redissolved

in 0.5 mL of chloroform and methanol (2:1, *v*/*v*), and aliquots were examined by TLC using the optimized TLC conditions that had been established empirically (see below), and the components were visualized using iodine vapor.

Purification of Cer(1,3)P by Silica Gel Column Chromatography

The Cer(1,3)P was separated from most of the other components of the reaction mixture by silica gel column chromatography with elution by chloroform and increasing proportions of methanol. The silica gel (Supelco) was suspended in chloroform and added rapidly to a small glass column (ca. 1 cm in width x 6 cm in height) with a glass frit until the settled bed of silica gel was approximately 3 cm in height. Then, the column was washed with several mL of chloroform and drained until the solvent reached the top of the column. Examination of the eluate showed that silica was not leaking through the frit, so without allowing the solvent to run dry at the top of the column, the reaction products (dissolved in ~ 0.5 mL of chloroform) were loaded to the column and washed in with several mL of additional chloroform. Fractions of approximately 3 mL each were collected in glass test tubes from the time that the reaction mixture was added to the column. In each step, 6 mL of the following solvent mixtures (chloroform with increasing % methanol) were added to the top of the column (with care not to disturb the silica) and the eluate from each collected in two test tubes; the % methanol were 0%, 1%, 5%, 25% and 50%.[*] Once all of the column fractions had been collected, the solvents were removed under a stream of N_2 with the test tubes in a tepid water bath, and then the contents were redissolved in 0.5 mL of chloroform:methanol (1:1, *v*/*v*). Aliquots from each of these tubes were spotted onto a silica gel TLC, along with standards and developed by the optimized TLC

[*] A better separation was later achieved by adding the methanol percentages 15% and 20%.

conditions that had been established empirically (see below), and the components were visualized using iodine vapor.

The TLC analysis revealed that the putative Cer(1,3)P appeared in the 25% methanol eluates, which also contained smaller amounts of Cer1P based on co-elution with the starting Cer1P and the intensity of the iodine staining. There was no contamination by the other major iodine-positive species (DCCD and N,N'-dicyclohexylurea), which eluted from the column earlier. To confirm that the major, higher R_f compound that appeared in the 25% methanol eluates was Cer(1,3)P, the material in these two test tubes was pooled and an aliquot (representing ~ 50 to 100 μg of Cer(1,3)P assuming > 50% of the starting Cer1P was converted to this product) was submitted to Dr. David Bostwick in the mass spectrometry core in the Petit Institute for Bioengineering and Biosciences for analysis by liquid chromatography-high resolution mass spectrometry.

TLC Optimization

The initial TLC conditions that were used had previously been reported to separate C6-NBD-Cer1P and C6-NBD-Cer(1,3)P,[53] which are more polar than the synthesized C24- species; therefore, both C24-Cer1P and C24-Cer(1,3)P migrated very high on the plate (R_f > 0.5). Thus, alternative solvents were tested to achieve better separation of the synthesis products. These solvents had varied components (using various combinations of two or more of the following: chloroform, methanol, acetic acid, water, and 2N ammonium hydroxide) in different ratios. The two best solvents for separation of C24-Cer1P and C24-Cer(1,3)P (and the corresponding C24-SM) were $CHCl_3$:methanol:H_2O:acetic acid (60:20:2:1, *v*/*v*/*v*/*v*) and $CHCl_3$:methanol:H_2O (60:20:2, *v*/*v*/*v*). Both of these solvents were accordingly used to analyze the synthetic products,

column eluates, and venom assay samples when the C24-sphingolipids were involved. It was noted that the substitution of ammonium hydroxide for acetic acid caused a large shift in the R_f of the C24-Cer1P (to nearly the same position as for SM) but not the Cer(1,3)P. This observation is consistent with the expected chromatographic behavior of Cer1P having a second dissociable hydroxyl with a pK_a that would enable it to be dianionic under these conditions.

Incubation of SMase D from Brown Recluse Spider Venom with SM

This assay was conducted with two substrates: C6-NBD-SM, which had been used by Dr. Merrill's previous students, and C24-SM, which has the advantage that there are now standards for the anticipated cyclic product from the synthesis described above. For the incubation, each assay contained 10 mM $MgCl_2$ and 100 mM Tris-HCl (pH 7.5), the SM substrate, and an uncertain quantity of SMase D from brown recluse spider venom (probably on the order of several ng, but the quantity was too low to assay) versus water for the no enzyme control. Since the SM requires a detergent to solubilize it, Triton X-100 and β-decyl-glucopyranoside were considered, and the latter was rejected because its R_f on TLC was too close to the SM. Both types of SM were highly soluble in Triton X-100, so substrate stocks were prepared at 1 mM (for C6-NBD-SM or C24-SM) with 1 mM Triton X-100, and these were diluted into the assay mixture for a final concentration of 100 μM.

The assay was conducted in 100 μL with half of the assay volume comprised of the SMase D and/or water and the rest for buffers and substrate. These tubes were capped and incubated in a dark 37 °C water bath overnight. The reaction products were extracted by organic solvents ($CHCl_3$:MeOH, 1:2, *v*/*v*), dried under a stream of N_2, and then redissolved in

$CHCl_3$:MeOH (2:1, *v*/*v*) for application to the TLC plate for separation using the aforementioned solvents.

Data analysis

No statistical analysis was conducted because the interruption of laboratory work at the pilot stage did not allow time to conduct the final assays in replicates. Therefore, the findings described in results are observations from pilot studies.

RESULTS

The first step of this project was to prepare Cer(1,3)P, the reported[40] product of the action of the brown recluse SMase D on SM, because this reagent is not currently available commercially. The published method[53] for synthesis of a fluorescent analog, C6-NBD-Cer(1,3)P, was not found to be successful because the Cer1P with natural N-acyl chains was not sufficiently soluble in DMF, even when a relatively short chain length (i.e., C12-) was used. However, this problem was solved by our finding that Cer1P is highly soluble in pyridine, which is another solvent that is often used for DCCD coupling reactions. The results of this reaction are presented below. The results are only described qualitatively because it was not possible to conduct enough replicate reactions to calculate a percentage yield due to the closing of the research laboratories mid-semester. Indeed, this was the case for all of the findings in this report; however, there were several clear deliverables from the work because we identified a reaction solvent (as mentioned) and found a TLC system that could separate Cer1P and Cer(1,3)P (as well as SM and Cer) for analysis of the synthetic reaction and for assays of SMase D. In addition, a preliminary experiment with brown recluse spider venom appeared show that it produced the cyclic phosphate, but this needs confirmation and quantitation. These results are described in more detail in the following subsections.

TLC Separation Conditions

Since there is not a method for separation of C24-Cer1P and C24-Cer(1,3)P has not been reported, we tried several variations of the protocol for separation of the NBD- analogs. These variations consisted of $CHCl_3$:MeOH:HOAc:15 mM $CaCl_2$ (60:20:1:2, *v*/*v*/*v*/*v*),[53] and we found that simpler mixtures of chloroform, methanol, and either water alone or with acetic acid

($CHCl_3$:MeOH:H_2O:HOAc, 60:15:2:1, *v*/*v*/*v*/*v* and $CHCl_3$:MeOH:H_2O, 3:1:0.1, *v*/*v*/*v*) were effective. Table 1 shows the R_f values for these compounds and SM and Cer under these conditions.

		Solvent		
		$CHCl_3$:MeOH:HOAc:15 mM $CaCl_2$ 60:20:1:2, *v* /*v* /*v* /*v*	$CHCl_3$:MeOH:H_2O:HOAc 60:15:2:1, *v* /*v* /*v* /*v*	$CHCl_3$:MeOH:H_2O 3:1:0.1, *v* /*v* /*v*
R_f Value	C24-Cer(1,3)P	0.44	0.46	0.48
	C24-Cer1P	0.34	0.28	0.19
	C24-SM	*	0.06	0.12
	C12-NBD-SM	*	0.06	0.16
	C12-NBD-Cer	*	0.95	0.93
		*compound has not been examined in given system		

Table 1. R_f values of Cer1P, Cer(1,3)P, SM, and Cer under various solvent conditions. A general trend, going from the bottom to the top of the TLC plate, appears to be SM, Cer1P, Cer(1,3)P, and then Cer resting closest to the top.

Column Chromatography Conditions

Column chromatography of the reaction products from synthesis of C24-Cer(1,3)P from C24-Cer1P using DCCD in pyridine was conducted as described under Methods. The unreacted DCCD eluted in the early fractions (chloroform and 1% methanol, not shown), whereas, the putative C24-Cer(1,3)P and unreacted C24-Cer1P eluted with 25% methanol (test tubes #10 and 11) as shown in Fig. 7.

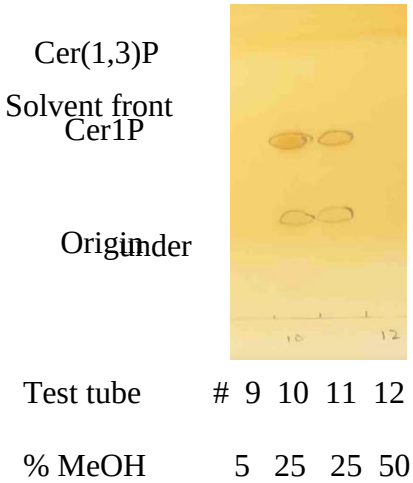

Figure 7. Compounds eluted from a silica gel column by 25% methanol in chloroform. Products from the synthesis of C24-Cer(1,3)P were applied to a small silica column as described Methods, and the fractions were examined by TLC with development by $CHCl_3$:MeOH:H_2O, 3:1:0.1, *v*/*v*/*v*.

A second column included intermediate methanol percentages, and C24-Cer(1,3)P was seen to elute in the test tubes for 15% and 20% MeOH. Therefore, a slight modification of this elution protocol should be able to get better separation of the Cer(1,3)P and unreacted Cer1P.

Evaluation of the Success of the Synthesis of Cer(1,3)P Synthesis in Pyridine

As can be seen in Fig. 7, the putative Cer(1,3)P was the darkest iodine staining material in test tube #10 (> 75% of the total) and appeared to be slightly darker or equal to Cer1P in test tube #11. Overall, the putative Cer(1,3)P in test tube #11 appeared to account for > 50% of the iodine staining; therefore, the reaction yield was at least that high.

To confirm that this material was Cer(1,3)P, an aliquot of the material in test tube #10 was analyzed by high resolution mass spectrometry. The negative ionization mode spectrum is shown in Fig. 8 and the structure and exact mass of C24-Cer(1,3)P is shown in Fig. 9 for comparison.

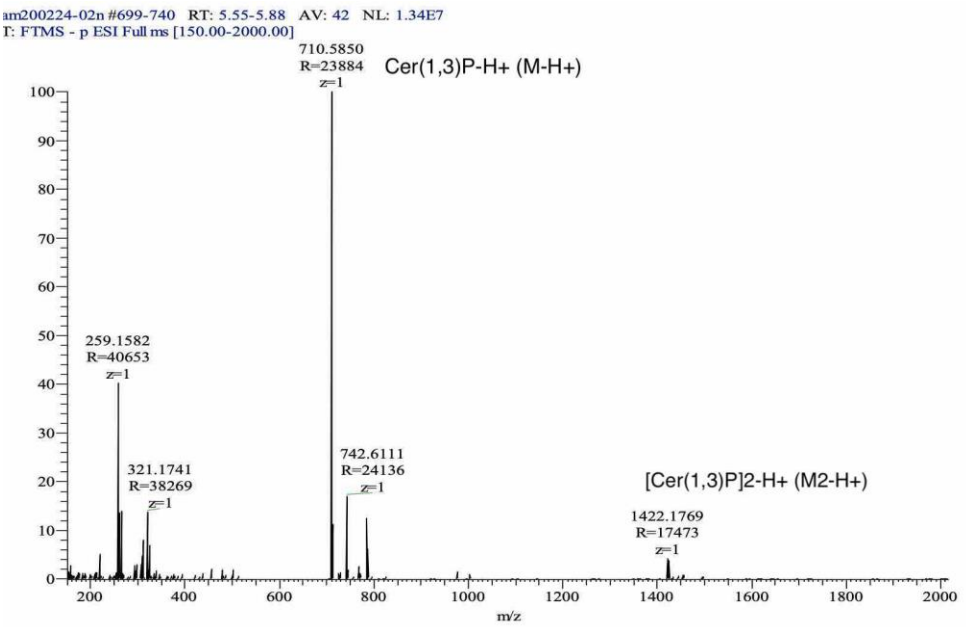

Figure 8. High resolution mass spectrum of sample #10 in negative ionization mode.

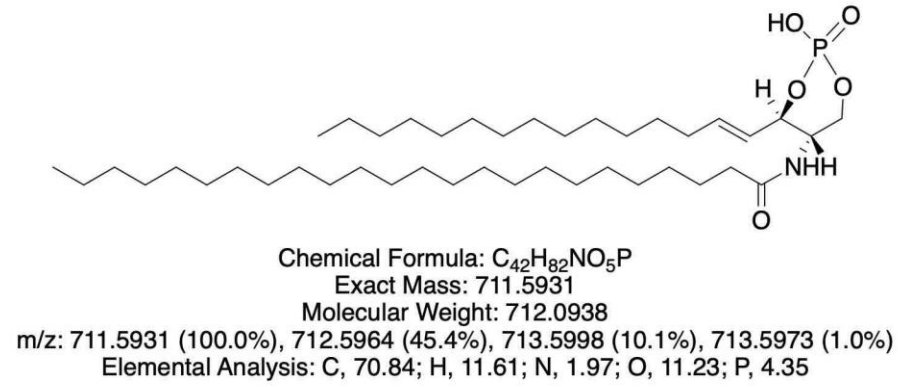

Figure 9. Chemical structure and exact mass of C24-Cer(1,3)P. This drawing and the calculated exact mass were prepared using ChemDraw Professional 16.0.

The major ion at *m*/*z* 710.5850 (Fig. 8) is consistent with the calculated exact mass of deprotonated Cer(1,3)P (i.e., M minus H^+ = 710.5858) (c.f., Fig. 9). In addition, the spectrum shows a minor peak at *m*/*z* 1422.1769 which is consistent with the predicted *m*/*z* for dimeric

Cer(1,3)P monoanion (i.e., 2 x M - H^+ = 1422.1789) (would be expected because lipids tend to form dimers in electrospray ionization MS).

Therefore, the identity of the C24-Cer(1,3)P was verified by mass spectrometry, and its migration position on TLC versus C24-Cer1P could be used to determine which product is produced by the brown recluse SMase D or other similar enzymes produced by other organisms.

Pilot Assay of Brown Recluse SMase D and Identification of the Product

Using the assay conditions described under Methods, C24-SM was incubated with purified SMase D from brown recluse spider venom and examined by TLC, as shown in Fig. 10. Although the photographic images are hard to see, it appeared to the eye that iodine staining was present in the regions for the substrate SM and the product Cer(1,3)Ps for the SMase D incubated tubes (Enz). The location of the Cer(1,3)P was established by the co-spotting with the synthetic column fractions #10 (which had only Cer(1,3)P) and #11 (which had Cer(1,3)P and Cer1P).

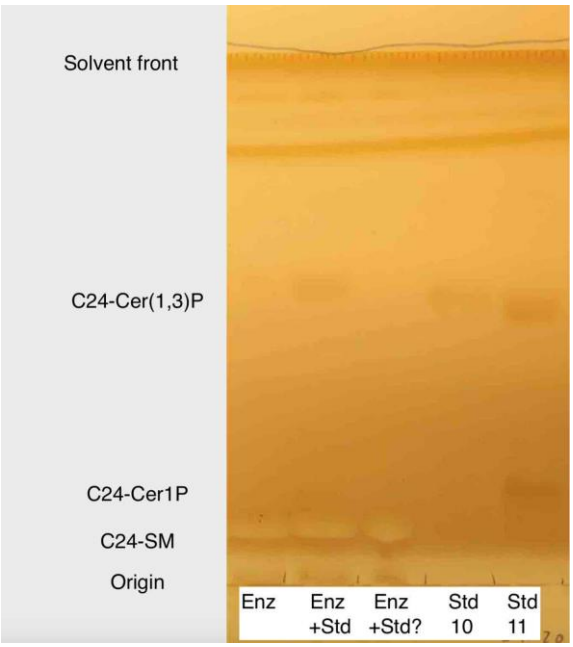

Figure 10. TLC of the lipid extracts after incubation of C24-SM with SMase D. The assays were conducted as described under Methods with the TLC plate developed with $CHCl_3$:MeOH:H_2O (3:1:0.1, *v*/*v*/*v*). "Enz" is the SMase D tube, "+Std" is the co-spotting of "Enz" with standards (column ftns #10 and 11). The std might have been omitted from "+Std?".

The purified SMase D from brown recluse spider venom was also incubated with NBD-SM as described under Methods, but the fluorescence was too faint for conclusions to be drawn. These analyses will need to be repeated and quantified, but the preliminary observations are promising.

DISCUSSION

The goal of this study was to develop a relatively easy method to analyze Cer(1,3)P to be able to determine with certainty when this product was made by SMase Ds from different organisms. Our plan was to begin with the brown recluse spider SMase D and eventually extend the method to other organisms.

The method was TLC-based since it does not require sophisticated instrumentation, and at least the fluorescent analogs (NBD) SM, Cer1P, and Cer(1,3)P had been separated by TLC in two previous studies.[40,53] But a limitation was the lack of a Cer(1,3)P standard to optimize and validate the method. That problem has now been solved, and when it is possible for work to continue in the lab, we will apply it to venoms from *Loxosceles* and other spiders.

Previous studies by students in Dr. Merrill's laboratory have identified that several other spiders have detectable SM hydrolytic activity in venom: *Kukulcania spp*. (YAAZ), *Larinioides spp*. (BVAZ), *Latrodectus tredecimguttatus*, *Psalmopoeus cambridgei*, and *Pterinochilus spp*. (Usambara). Therefore, it is important to determine if they produce the same SMase D product as *Loxosceles* venom (i.e., Cer(1,3)P) or perhaps the product of mammalian sphingomyelinases and other enzymes (i.e., Cer1P and/or Cer).

This could be significant clinically if the production of Cer(1,3)P is a possible cause of dermonecrotic lesions. Since *Loxosceles* bites are quite difficult to accurately assess at the moment, especially if the culprit spider is not available to be identified by an expert, this assay could help to alleviate the identification problem somewhat. We could also find that perhaps not all necrotic wounding possesses SMase D activity. Ultimately, this assay would hopefully lead to better, faster, and more targeted recovery of affected individuals.

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